Reprinted from AMERICAN JOURNAL OF CLINICAL PATHOLOGY, Vol. 77, No. 5, May 1982

Localization of Legionella pneumophila *in Tissue Using FITC-Conjugated Specific Antibody and a Background Stain*

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Lowry, Barbara S., Vega, Filiberto G., Jr., and Hedlund, Kenneth W.: Localization of *Legionella pneumophila* in tissue using FITC-conjugated specific antibody and background stain. Am J Clin Pathol 77: 1982; 601–605. Lightly staining formalin-fixed or fresh tissue with Gram's crystal violet obviates interfering nonspecific fluorescence by acting as a metachromatic stain in ultraviolet light. Against the easily recognized background of tissues and cells fluorescein isothiocyanate-tagged *Legionella pneumophila* antibodies can then identify this bacterium in or on individual cells. This procedure can be run at room temperature in two hours and has the potential for further widespread applicability. (Key words: *Legionella pneumophila*; Gram's crystal violet; FITC-tagged antibodies)

Received June 19, 1981; received revised manuscript and accepted for publication July 31, 1981.

In conducting the research described in this report, the investigators adhered to the "Guide for the Care and Use of Laboratory Animals", as promulgated by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resourses, National Research Council. The facilities are fully accredited by the American Association for Accreditation of Laboratory Animal Care.

The views of the authors do not purport to reflect the positions of the Department of the Army of the Department of Defense.

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USING THE COMMON stains to identify bacteria in tissue, *Legionella pneumophila* typically stains poorly, if at all, and frequently with baffling inconsistency.[1,2] Legionella can, however, be easily identified in ultraviolet light (UV) with a fluorescein isothiocyanate (FITC)-tagged specific antibody. In searching for *L. pneumophila* in tissue in the fluorescent mode, background autofluorescence often interferes with identification of the organism, while absence of background staining leaves no idea as to the site of the bacterium in the tissue. Various dyes, including Evans blue, while quenching background autofluorescence fail to give the desired tissue counterstain. We have found that formalin-fixed and processed tissue lightly stained first with Gram's crystal violet can then be reacted with the FITC-tagged specific antibody to pin-point *L. pneumophila*, either free or in phagocytes, against an easily identified histologic background.

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Materials and Methods

Background Stain

Ordinary Gram's crystal violet stains both formalin-fixed tissue and frozen sections and subsequently causes tissue metachromatic fluorescence under UV (reagents from a commercial Gram set* compare favorably with a common formulation for the crystal violet and acid-alcohol decolorizer[5]). Originally we used Gram's crystal violet directly from the dropping bottle, immediately followed by washing with distilled water and decolorizing to a pale blue-violet color with Gram's acid-alcohol. After experimenting we found that a 1:100 dilution of Gram's crystal violet stain in distilled water was optimal. A rapid dip of the slide in this stain followed by an immediate rinse with distilled water tended to yield a pale color, and frequently necessitated no decolorization.

Tissue sections were cut at 3 μm; thicker sections tended to take up more stain and required greater decolorization. Pale stains allowed antigenic sites to remain available on *L. pneumophila* for combination with specific antibody, while heavier stains tended to interfere with the antigenic sites leaving no reactive foci for combination with the FITC-tagged specific antibody and a resultant tissue section falsely negative for *L. pneumophila*. Positive and negative controls were run with each slide or batch of slides processed and stained.

Tissues from AKR/J and Vesper mice and Hartley strain guinea pigs were obtained from test animals infected with *L. pneumophila* by aerosolization or intraperitoneal injection routes. Uninfected animals served as controls.

Cultures and Antisera

Philadelphia (L_1, L_2, L_4), Knoxville$_1$ (serogroup 1) and Bloomington$_2$ (serogroup 3) strains of Legionella were tested. These were originally obtained from the Centers for Disease Control (CDC), Atlanta, Georgia, and stored frozen as seed-stock from embryonated hens' eggs and/or passed on charcoal yeast extract (CYE) agar. All reacted with appropriate FITC-conjugated specific antisera, and were grown on CYE agar prior to animal infection.

Both CDC's mono- and polyvalent FITC-conjugated specific antisera to *L. pneumophila* and FITC-conjugated monovalent specific antiserum produced at this Institute were used to identify and locate *L. pneumophila* in tissue sections.

* #3328-32-1, Difco Laboratories, Detroit, Michigan.

Tissue Preparation

We experimented with several methods[3,4,6,7] for preparing formalin-fixed tissue for trypsinization and subsequent direct fluorescent antibody (FA) reactions and elected a modification which was carried out at room temperature (RT) and completed in just under two hour's time (Table 1).

Trimmed, formalin-fixed tissue was submitted in tissue capsules to histopathology for processing and cutting at 3 μm. Sections were floated in a 2-liter, 46°C water-bath containing about 0.2 g of gelatin† sprinkled over its surface and dissolved, to cause the specimens to adhere to the acetone-cleaned glass slides. The slides were then held overnight in a slide warmer at 46°C and stored in slide boxes at RT.

Paraffin was removed from tissue sections by running the slides through xylol, graded alcohols and a distilled water wash. The tissue was then trypsinized and washed in cold buffered phosphate saline (PBS),‡ although we have found distilled water to work well in most cases, avoiding the residue from the buffer. At this stage slides can be stored at 4°C for future use.

Staining

The tissue was then stained with Gram's crystal violet (or diluted stain) and, if necessary, immediately decolorized to a pale blue-violet with acid-alcohol. The aim was a *pale* blue-violet color imparted to the tissue by means of the dye and destaining by acid-alcohol as necessary, not the absence of color. Destaining was accomplished as with the usual gram-stain procedure for bacteria: solvent was dropped on the tissue until the liquid running off the slide was water clear. Rarely, complete color removal occurred and then restaining was necessary with less drastic decolorization. Each slide demanded individual attention, but the staining and destaining procedure was rapid and usually complete in 30–45 seconds. The slide was then washed in distilled water, air-dried and overlaid with FITC-conjugated specific antiserum in a moist chamber for 20 min.

Applying a glycerol mounting medium (18 ml glycerine and 2 ml PBS) and a cover slip, the finished tissue slide was stored in a folder protected from light and could be viewed immediately or over the next few days.

Microscopic Examination

A Zeiss microscope, equipped for epifluorescence, with exciter-barrier filter and reflector combination

† Fischer Scientific Co., Fairlawn, New Jersey.

‡ Buffer: $NaH_2PO_4H_2O$, 2.76 g; NaCl, 16.00 g; Distilled water q.s., 2.0 liters; Adjust *p*H to 7.2–7.4 with 1 N NaOH.

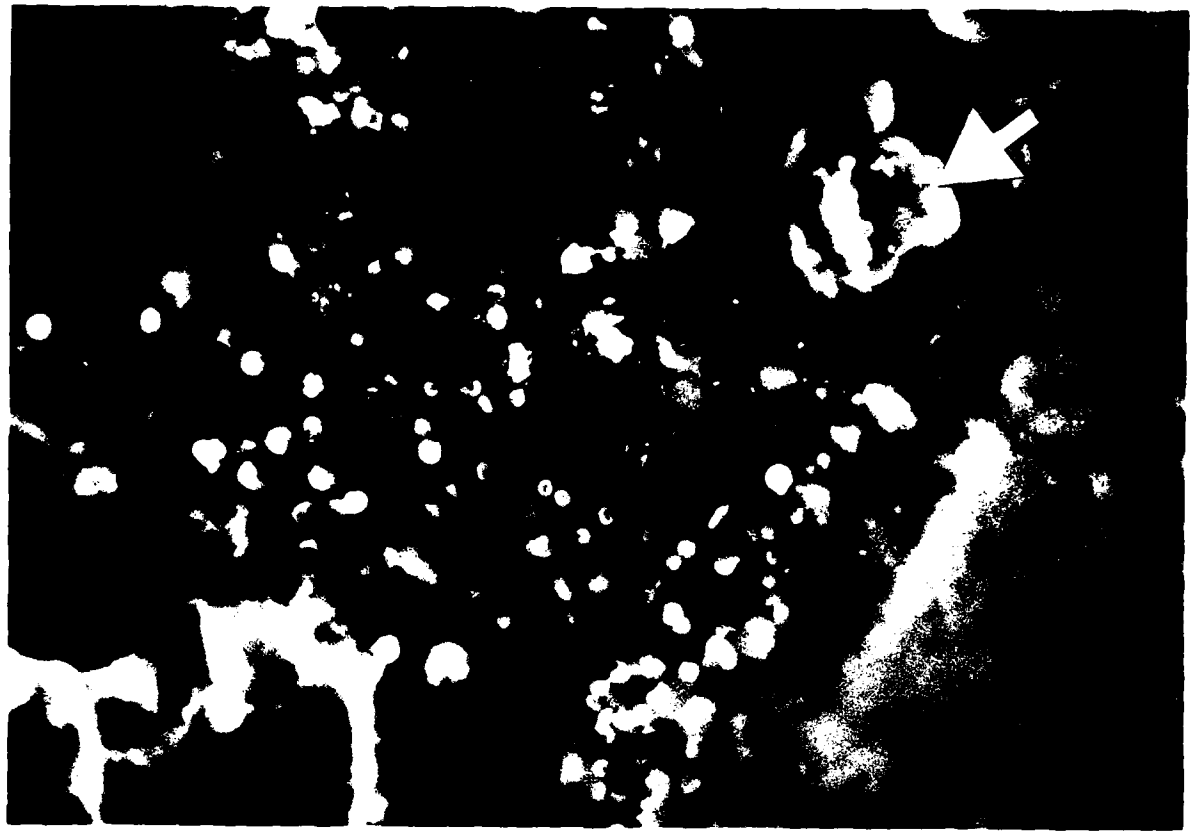

FIG. 1 (*upper*). Mouse peritoneal tissue 24 hours after IP injection with *L. pneumophila*, stained for background and immunofluorescence. On right is fibrous tissue, staining brownish-orange with a blood vessel in the upper right quadrant (arrow). Loose, areolar tissue takes a tannish to green stain. Free *L. pneumophila* stain a light apple green and tend to be clustered centrally. Phagocytic cells, mostly macrophages, have an orange-red nucleus and are bright yellow when occupied by *L. pneumophila* or its antigenic debris. Red blood cells are a pale orange, but easily identifiable by size and concave shape. Original magnification, ×400.

FIG. 2 (*lower*). Guinea pig lung showing acute phase of pneumonic Legionellosis. The alveolar lumen is packed with exudate composed of phagocytic cells and fibrin. *L. pneumophila* can be identified free and within phagocytes. The blood vessel (arrow) contains antigenic fragments both within a phagocyte and adherent to the vessel wall. Original magnification, ×400.

Table 1. Procedure to Localize *Legionella pneumophila* in Tissue Sections Using a Background Stain and FITC-Conjugated Specific Antibody

1. Preparation of formalin-fixed, processed tissue, cut 3 mμ thick, glass slide.
   a. Deparaffinization
      1) Xylene bath 2 3 min
      2) Xylene bath 2 3 min
   b. Rehydration
      1) 99% EtOH bath 15 sec
      2) 95% EtOH bath 15 sec
      3) 50% EtOH bath 15 sec
      4) Rinse in distilled water 2 3 min
   c. Trypsinization
      1) 0.25% trypsin in distilled water 1 hour
      2) Rinse in PBS, *p*H 7.2 7.4, 5 min (can store slides at 4°C)
2. Staining for formalin-fixed and frozen sections with Gram's crystal violet.
   a. Overlay about 5 sec or one dip in staining jar
   b. Rinse with distilled water
3. If necessary, decolorize with Gram's acid-alcohol until a pale blue-violet
4. Air-dry
5. Overlay with FITC-conjugated specific antiserum in moist chamber 20 min
6. Rinse in distilled water
7. Mount in mixture glycerol medium
   a. Coverslip for viewing
   b. Store covered and/or in dark

FITC blue,§ excitation band pass filter 450–490 nm and barrier filter 520–560 nm, was used for viewing specimens. With 10× oculars and 40/0.85 and neofluor phase 100/1.30 objectives, good results were obtained even in a lighted room with the microscope unshielded. The accompanying photographs, enlarged film transparencies of Kodak Ektachrome 160 tungsten film,¶ show the type of results that can be expected (Figs. 1 and 2).

Tissue Appearance

The staining procedure tends to impart a red-orange color to tissue in UV light, but with a properly pale stain the dye appears multi-hued, with respiratory epithelium tan to red-orange, connective tissue yellow to brown, red blood cells bright to deep red (occasionally yellow), cartilage greenish, and muscle red to red-brown. The apple green fluorescence of the FITC-antibody *L. pneumophila* conjugate and the golden yellow color typical of the tagged phagocytized organism and its antigenic fragments stand out against the contrasting background colors of the tissue. The metachromatic stain furnishes surprisingly good detail in UV light, and a quick microscopic adjustment can change our lighting to UV and white (in which the tissue is tannish) or white light alone, illuminating the pale blue to violet stained section for comparison with the appearance in UV light.

§ Carl Zeiss #487710.
¶ Eastman Kodak, Rochester, New York.

Trials with Other Microorganisms

The technic described for locating *L. pneumophila* in tissue works with other microorganisms, but is limited if the antigenic sites are altered by formalin (somewhat analagous to toxoiding of diphtheria and tetanus toxins) in fixation. The use of other tissue fixatives may overcome this drawback. A trial treatment of a spot slide of a specific organism with formalin followed by staining with FITC-conjugated specific antiserum can establish whether this technic is applicable. In our hands *Staphylococcus aureus* stained brilliantly, *Escherichia coli* lost some brilliance, and *Neisseria gonorrhoeae* completely failed to stain with the homolgous antibody after fixing in formalin. Rift Valley fever virus and its specific FITC-conjugated antiserum stained infected whole cells brightly.

Three months after staining, a surprising amount of fluorescence can be restored by destaining with acid alcohol, and repeating the staining process. Thick tissue or thicker areas in tissue tend to take up more crystal violet, stain a more intense blue-violet and consequently appear a deeper red-orange in UV light, and make binding sites unavailable for the FITC-coupled antiserum. These slides, too, can be destained and carefully and more lightly restained before incubating with the fluorescent-tagged antiserum again. Gentian violet gave results comparable to staining with the crystal violet.

Summary

L. pneumophila can be located in tissue and cells rapidly and easily using the first two gram-stain reagents and FITC-tagged homologous antibodies.

The technic suggests far-reaching applications in fields other than microbiology. Since fresh tissue takes up the stains similarly to fixed tissue, frozen sections can also be stained by this technic for a rapid, one-slide identification of antibody receptors and their locations in tissue and cells. Although our work to date has been with the direct FA technic only, this procedure should work equally well with the indirect FA technic.

NOTE

Since submission of this article we have instituted a quick dip of the deparaffinized, trypsinized tissue slide in 0.1 N HCl before dipping in gram's crystal violet, diluted 1:100 with distilled water. These changes give more uniform results and eliminate the decolorization step.

Acknowledgments. We wish to thank Dr. Richard F. Berendt for his technical support in the aerosolization of *L. pneumophila* and James C. Hardy, Histology Technician, whose patience and high level of technical accomplishment made this research possible.

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